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Dual inhibition of the dactyl opener muscle in lobster

Mary Kate Worden* and Joseph A. Camacho
Department of Neuroscience, University of Virginia, PO 801392, Charlottesville, VA 22908, USA
*Author for correspondence (e-mail: mkw3k@virginia.edu)

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Summary

The dactyl opener neuromuscular system of crayfish and lobster has long been a popular model system for studies of synaptic physiology and its modulation. Previous studies of its neural innervation in both species have reported that whereas the opener excitor axon (OE) and the specific opener inhibitor (OI) innervate the entire muscle, the common inhibitor (CI) is restricted to a small number of the most proximal muscle fibers and is the physiologically weaker of the two inhibitors. Here, we show in the lobster that, contrary to previous reports, CI innervates fibers along the entire extent of the dactyl opener muscle and thus shares the innervation targets of OE and OI. To characterize the physiological function of CI in the lobster dactyl opener system, we independently stimulated both OI and CI and compared their effects. The physiological impact of each inhibitor was similar: inhibitors elicit inhibitory junction potentials of similar sizes, both trigger muscle relaxations and both inhibit excitatory junction potentials and muscle contractions to a comparable extent. Thus, in the periphery, CI and OI appear to be equally powerful inhibitors with similar physiological roles, both in suppressing contractions triggered by the excitor OE and in directly relaxing muscle tension. In light of these observations, our understanding of the physiological roles of specific and common inhibitors in crustacean motor control requires reevaluation.

Key words: inhibition, dactyl opener, lobster, crustacean, CI, common inhibitor, *Homarus americanus*.

Introduction

The dactyl opener muscle of decapod crustaceans has long served as a model system for physiological studies of the cellular mechanisms underlying synaptic transmission and its modulation (e.g. Bykhovskaia et al., 1999; Bykhovskaia et al., 2004; Dudel and Kuffler, 1961; Parnas et al., 2000; Vyshedskiy et al., 1998; Vyshedskiy and Lin, 2000; Worden et al., 1995; Worden et al., 1997; Zucker, 1973). In part, the popularity of this neuromuscular system among physiologists arises from the simplicity of its neural innervation. In astacuran species (lobsters and crayfish), neural innervation is supplied both by a specific opener excitor axon (OE) and a specific opener inhibitor axon (OI) as well as a common inhibitor axon (CI) that also supplies inhibition to all the other limb muscles (reviewed in Wiens, 1989). It has been well established that the innervation patterns of OI and OE motoneurons are coextensive; the muscle fibers of the opener are 100% tonic (Mearow and Govind, 1986), and OI and OE supply all of them (Govind, 1995; Marmont and Wiersma, 1938; Van Harreveld, 1939).

However, the extent to which CI innervates the fibers of the dactyl opener is not clear. In the dactyl opener of the crayfish *Procambarus clarkii*, CI elicits synaptic responses only in a small number of the most proximal muscle fibers and exerts a weaker physiological effect than does OI, suggesting that CI does not innervate the bulk of the opener muscle (Wiens, 1985; Wiens, 1989). This type of restricted innervation pattern contrasts sharply with the arrangement in the brachyuran crab species *Eriphia*, where CI innervates all the fibers of the dactyl opener muscle (Wiens et al., 1988). In the lobster *Homarus americanus*, Wiens confirmed dual inhibitory innervation of the dactyl opener by both OI and CI for a small number of fibers in the proximal region (Wiens, 1990). However, that study did not examine whether CI, like OI, makes synaptic contacts along the entire proximal–distal extent of the muscle.

The issue of whether CI does, in fact, supply only a small region of the muscle is an important consideration for understanding its functional role in astacurans. The physiological role of the specific inhibitor OI has always been considered in the context of the excitor OE, with which it is closely associated anatomically and with which it shares all innervation targets. OI exerts both postsynaptic and presynaptic inhibition in the dactyl opener system (reviewed in MacDermott et al., 1999). Since OE supplies the dactyl opener as well as the stretcher muscle, it seems likely that OI serves to moderate OE signaling to the opener and enable independent function by the stretcher, as first suggested by Marmont and Wiersma (Marmont and Wiersma, 1938). By contrast, the physiological role of CI is less clear. Its physiological effects are reported to be weak compared with

those of OI (Wiens, 1985; Wiens, 1993), however as it supplies all limb muscles it is thought to facilitate alternating contractions of antagonistic muscle pairs (Ballantyne and Rathmayer, 1981; Wiens, 1989).

In the present study, we examine the innervation pattern of CI in the lobster dactyl opener and compare the physiological effectiveness of CI and OI in inhibiting the dactyl opener neuromuscular system. In contrast with previous studies on decapod crustaceans, we find that CI innervates fibers along the entire length of the dactyl opener. Further, we find the physiological impact of CI and OI to be strikingly similar: both relax muscle to a comparable extent, and each inhibits synaptic responses and muscle contractions stimulated by the excitatory motoneuron OE.

Materials and methods

All experiments were performed on lobsters (*Homarus americanus* H. Milne Edwards 1837) that had been acclimated for a month or more to artificial seawater at a temperature of 4–6°C. Dissections and experiments were performed in *Homarus* saline (462 mmol l^{-1} NaCl, 16 mmol l^{-1} KCl, 26 mmol l^{-1} $CaCl_2$, 8 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} Hepes, 11 mmol l^{-1} dextrose, pH 7.4). During dissection under chilled saline, the axons of OE and OI as well as a nerve branch to the closer muscle containing the axon of CI were identified by following each proximally from the nerve innervating the dactyl opener. The preparation was then transferred to a recording chamber enclosed in a resin block through which refrigerated coolant flowed from a refrigerated circulator. The neuromuscular preparation was constantly superfused with saline at 3–5 ml min^{-1} and the temperature of the bath was monitored and maintained at 2°C. In some experiments, the temperature was warmed from 2 to 22°C by heating the coolant and the saline reservoir.

Methods for intracellular recording of synaptic potentials are as described previously (Worden et al., 1995). Each of the three axons innervating the dactyl opener was drawn into a separate suction electrode and stimulated (stimulator model S88; Grass-Telefactor, West Warwick, RI, USA) with brief (1–3 ms) stimuli sufficient to elicit synaptic responses in the dactyl opener muscle. In the carpus, the axon of the opener motoneuron OE was stimulated *via* the nerve that innervates the stretcher muscle (arrow 1, Fig. 1), and the axon of the inhibitor OI was stimulated by dissecting the OI axon from the main nerve trunk (arrow 2, Fig. 1). The axon of the inhibitor CI was stimulated in the propus *via* a nerve branch that innervates the dactyl closer muscle (arrow 3, Fig. 1) (Wiens, 1990). Intracellular recordings of synaptic potentials in the muscle fibers of the dactyl opener were made using glass microelectrodes filled with 3 mol l^{-1} potassium acetate and a Neuroprobe (A-M Systems, Carlsborg, WA, USA) amplifier and filtered at 100 Hz. At 2°C, synaptic responses to OE were depolarizing, while responses to OI and CI were hyperpolarizing. In every recording we verified that stimulation of OI and CI generated synaptic responses that could be distinguished by their shape and/or size. In this manner, we confirmed that both axons innervated the muscle fiber and ruled out the possibility that we inadvertently stimulated release from one of the inhibitors twice. Muscle resting potentials at 2°C were in the range of –51 to –89 mV. Muscle tension was measured by tying 6.0 silk surgical thread to the distal end of the apodeme of the dactyl opener and attaching the thread to a Grass FT03 force transducer (Grass-Telefactor). Tension recordings were filtered at 10 Hz and amplified by a Cyberamp (Axon Instruments–Molecular Devices, Union City CA, USA) and calibrated in units of grams. All data were recorded on a VCR, digitized by an analog-to-digital converter and analyzed using pClamp

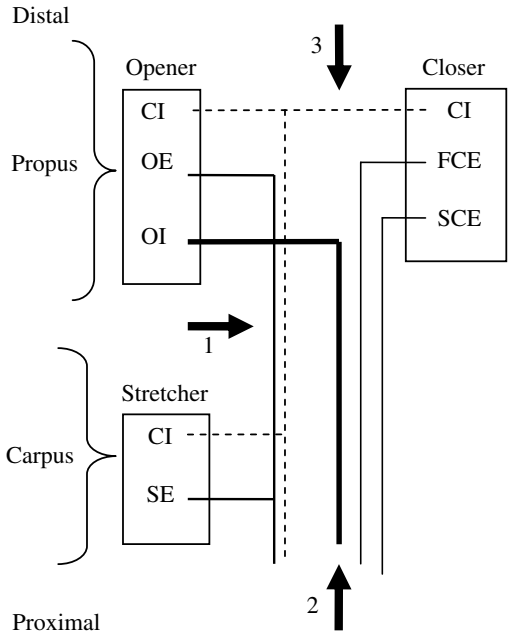

Fig. 1. Schematic illustrating the neural innervation of the dactyl opener and closer muscles of the propus and the stretcher muscle of the carpus of the lobster walking leg. Muscles are indicated by boxes; within the boxes are abbreviations for the names of the innervating motoneurons. Lines illustrate the innervation paths of the axons of each of the motoneurons. The opener muscle receives innervation from the common inhibitor (CI), the opener excitor (OE) and the specific opener inhibitor (OI). Placement of suction electrodes for stimulation of the axons of these motoneurons is illustrated by numbered arrows. (1) The axon of OE is stimulated by an electrode placed on the nerve running between the stretcher and opener muscle; this nerve contains the axon of OE (black line) as well as CI (see Results). (2) The axon of OI is stimulated *via* its axon (heavy black line) in the main nerve trunk in the carpus. (3) The axon of CI (broken lines) is stimulated by an electrode placed on a nerve branch that stretches between the opener and closer muscles. The innervation of the closer muscle and the stretcher muscle is also shown: the closer is innervated by fast and slow excitatory axons (FCE and SCE) as well as by CI, while the stretcher is innervated by CI as well as the stretcher excitor (SE). The stretcher excitor SE is another name for the opener excitor OE; both muscles are innervated by the same excitatory axon.

software (Digidata1200 A-D converter and pClamp software from Axon Instruments–Molecular Devices). Means of measurements are reported ± s.e.m., unless otherwise indicated.

To compare the effectiveness of each of the inhibitors in inhibiting synaptic transmission by the excitor OE, one of the inhibitory motoneurons (either OI or CI) was fired with a delay ($-150 \text{ ms} < x < 800 \text{ ms}$) relative to stimulation of the OE. The size of the excitatory junction potential (EJP) elicited by OE was then measured with respect to the muscle resting potential prior to both stimuli. Experimental trials where the inhibitor was stimulated were alternated with control trials in which the inhibitor was not stimulated. In cases where presynaptic inhibition was measured by analysis of area, the area under the EJP was found by integrating the area under the curve described by the EJP with respect to the baseline determined as the level of the resting membrane potential prior to stimulation.

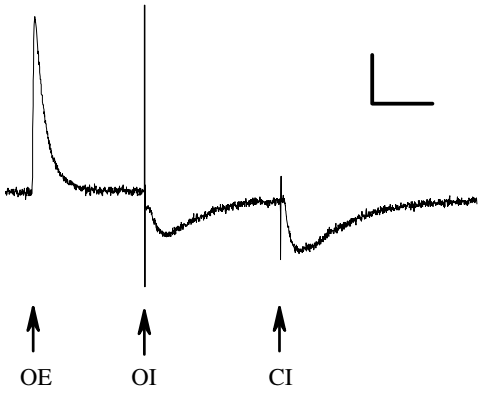

Fig. 2. Synaptic responses recorded in a dactyl opener muscle fiber in response to stimulation of the opener excitor (OE), the specific opener inhibitor (OI) and the common inhibitor (CI) motoneurons. The relative timing of stimulation for each motoneuron is indicated schematically. Muscle potential was –70 mV. Scale bar, 1 mV, 250 ms.

Results

CI innervates muscle fibers all along the proximal–distal axis of the muscle

Three motoneurons innervate the lobster dactyl opener: the OE, the OI and the CI. Fig. 2 shows an intracellular recording from a dactyl opener muscle fiber in which all three motoneurons elicited synaptic responses. This recording is typical in that, at 2°C, the inhibitory junction potentials (IJPs) triggered by OI and CI are hyperpolarizing in sign and differ slightly in terms of their amplitude and time course, while the EJP elicited by OE is depolarizing. All three synaptic responses appear prolonged (>200 ms), most likely because cold temperatures slow the kinetics of neurotransmitter release and affect muscle membrane properties.

To determine the innervation pattern of CI along the dactyl opener muscle, intracellular recordings were made from a series of muscle fibers located at different positions along the length of the muscle. Stimulating the CI axon triggered IJPs in fibers all along the proximal–distal axis of the muscle (Fig. 3).

Because the inhibitory neurotransmitter at crustacean neuromuscular synapses is thought to be GABA (reviewed in Atwood, 1972), we tested whether synaptic responses to both CI and OI would have the same reversal potential, as would be predicted if they release the same neurotransmitter. Warming the preparation from 2 to 22°C produces hyperpolarization of the muscle fibers and reverses the sign of the inhibitory synaptic responses from hyperpolarizing to depolarizing (Fig. 4). Table 1 shows the results from experiments on multiple fibers. Synaptic responses triggered by each inhibitor reversed polarity at similar membrane potentials and bath temperatures, consistent with the idea that both inhibitors release the same neurotransmitter.

To test the possibility that CI reaches the dactyl opener *via* the nerve that carries the axon of the stretcher/opener excitor to the stretcher and opener muscles, a series of stimulation experiments were performed. Stimulation of the nerve supplying both the stretcher and the opener muscles (arrow 1, Fig. 1) elicited EJPs at low stimulus voltages by recruiting OE (see Materials and methods). However, higher stimulus voltage

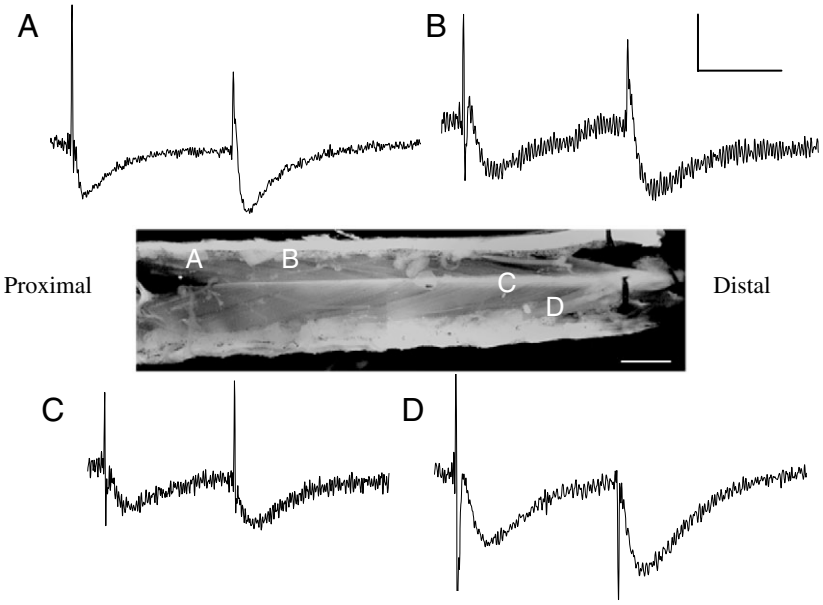

Fig. 3. Synaptic responses elicited by both the opener inhibitor (OI) and the common inhibitor (CI) can be detected in many regions of the dactyl opener muscle. Intracellular recordings from different areas of the muscle (labeled A–D) are shown. In each case, CI was stimulated before OI. Scale bars for electrophysiological recordings, 2.5 mV, 0.5 s. Scale bar for photo, 0.25 cm.

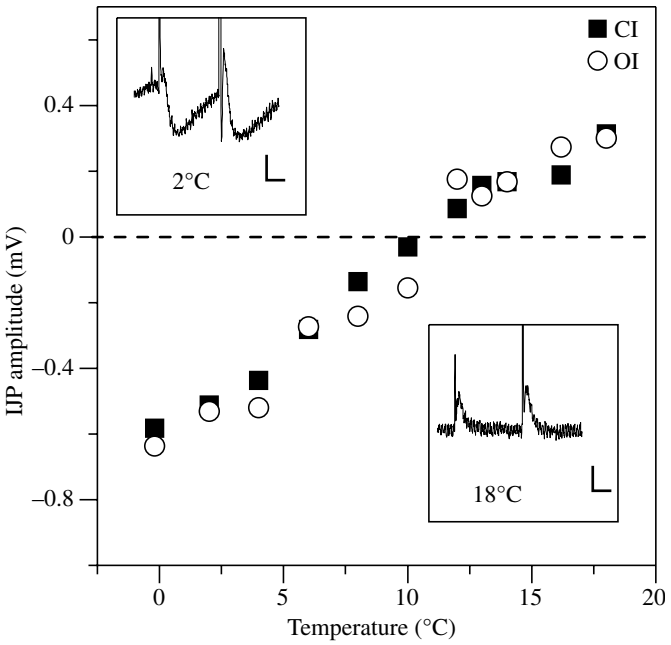

Fig. 4. Synaptic responses elicited by the opener inhibitor (OI) and the common inhibitor (CI) reverse polarity at similar temperatures. The amplitudes of inhibitory junction potentials (IJPs) elicited by stimulation of OI and CI are plotted as a function of temperature. All measurements were made in a single muscle fiber; insets show synaptic responses at 2°C and 18°C. Note that IJPs are hyperpolarizing at colder temperatures (<10°C) and depolarizing at warm temperatures (>12°C). Scale bars, 0.5 mV, 0.2 s.

recruited a combination of IJPs and EJPs (data not shown), consistent with the possibility that the axon of CI travels with the axon of OE to reach both the stretcher and the opener before branching to reach the closer (path illustrated by broken line in Fig. 1).

Finally, to obtain histological confirmation that three axons innervate muscle fibers throughout dactyl opener preparation, we stained preparations with methylene blue. While it was rare to find regions where all three axons were apparent, Fig. 5 shows two examples where three axons could be identified within the nerve innervating the dactyl opener. In both cases,

Table 1. *The reversal potentials of the inhibitory junction potentials (IJPs) elicited by the common inhibitor (CI) and opener inhibitor (OI) in dactyl opener muscle are similar*

Inhibitor	Muscle fibers (*N*)	Reversal potential (mV)	Temperature (°C) at IJP reversal potential
CI	10	–65.70±9.36	10.49±3.99
OI	9	–63.89±10.58	10.85±4.44

Values are means ± s.d. The membrane potential at which the IJP reversed polarity was measured intracellularly as temperature was increased from 2 to 22°C. In five of the experiments, CI and OI were monitored in the same muscle fiber.

a small-diameter axon could be seen running with two larger-diameter axons and branching alongside them.

CI and OI are equally effective at hyperpolarizing the muscle fibers and relaxing the muscle

To compare the physiological impact of the inhibitory neurotransmission from the CI motoneuron with that of the OI motoneuron, we measured the sizes of IJPs elicited by both in single muscle fibers. Overall, IJPs elicited by OI were significantly larger than those elicited by CI [0.80±0.09 mV (mean ± s.e.m.) compared with 0.64±0.07 mV; $P<0.05$; paired sample *t*-test; $N=34$]. However, the magnitude of this difference is not large, on the order of 20–25%. Calculated in each of the 34 fibers as a ratio (CI:OI), the mean ratio was 0.90. In 11 of the 34 fibers tested, the IJPs resulting from stimulation of CI were larger in size than those resulting from OI. Measurements of IJP size were internally consistent among the fibers within a single neuromuscular preparation: IJPs from OI were larger than those from CI in each of multiple muscle fibers ($N=11$) from three preparations, while in a fourth preparation CI elicited larger IJPs than did OI in all fibers tested ($N=9$).

To compare the effectiveness of each motoneuron in influencing muscle tonus, we stimulated OI and CI and monitored the resulting changes in muscle tension. Fig. 6A shows an example of a preparation in which single IJPs

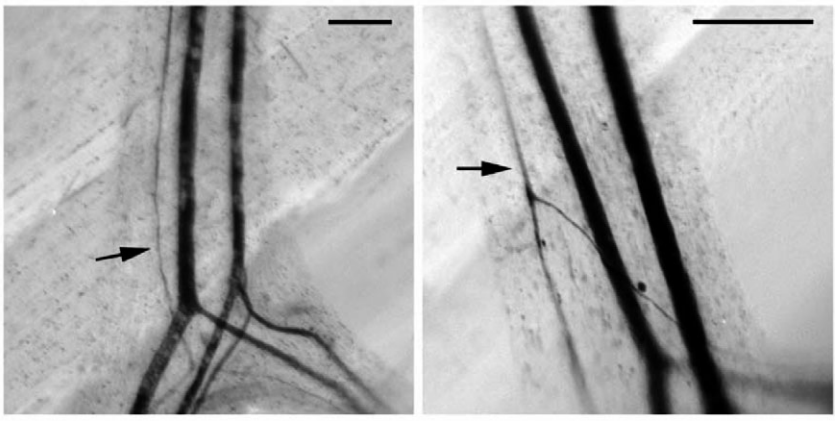

Fig. 5. Methylene blue staining reveals three axons in the nerve innervating the dactyl opener. Left and right photos were obtained from sites located 25% and 75%, respectively, along the length of the entire proximal–distal axis of the dactyl opener muscle. Proximal is at the top of each photo. Arrows show a small-diameter axon that branches (as do two large diameter axons) distally to supply branches of the nerve. Scale bars, 50 μm.

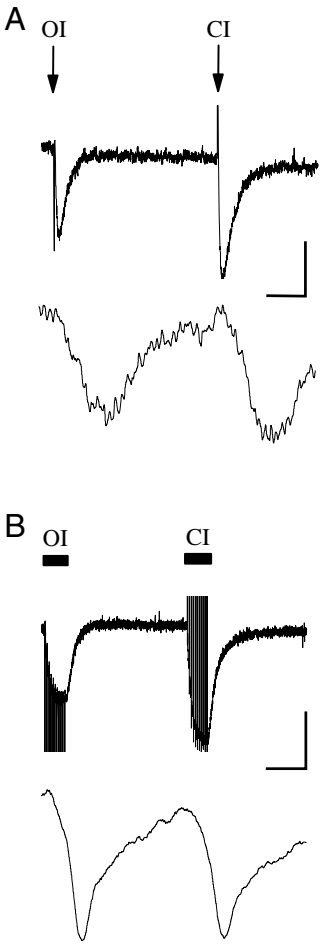

Fig. 6. Inhibitory synaptic responses and muscle relaxations triggered by the opener inhibitor (OI) and the common inhibitor (CI). (A) Upper trace shows inhibitory junction potentials (IJPs) elicited by single stimuli (indicated by arrows) to OI and to CI; lower trace shows the corresponding relaxation in muscle tension. Scale bar, 0.1 mV, 4 mg, 1 s. (B) Upper trace shows synaptic responses to identical 10 Hz trains of stimuli delivered to OI and CI; lower trace shows the corresponding relaxation of muscle tension. The timing of stimulus trains is indicated by horizontal black bars. Scale bar, 0.5 mV, 12.5 mg, 2 s.

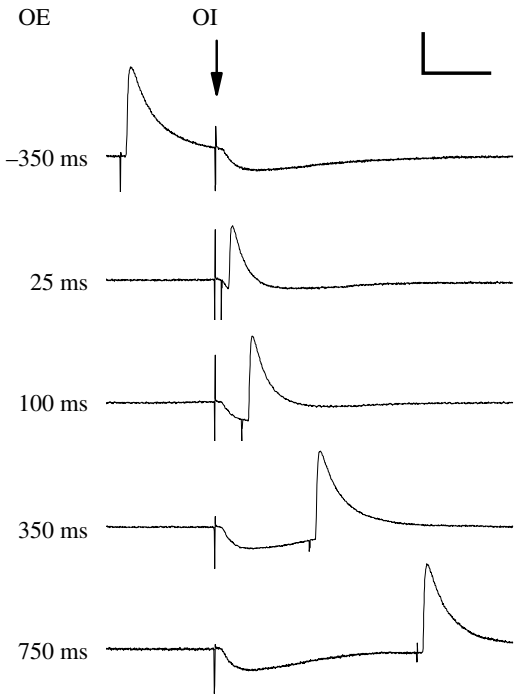

Fig. 7. Inhibition of excitatory junction potentials (EJPs) by the opener inhibitor (OI) depends on interstimulus interval. A series of recordings from a single muscle fiber is shown; each trace is the average of six trials. In each recording, OI is stimulated at the time indicated by the arrow, the opener excitor (OE) was stimulated at an interstimulus interval indicated to the left of each trace. Note that the delay is preceded by a negative sign if OE is stimulated before OI. Scale bar, 2.5 mV, 250 ms.

triggered muscle relaxations; both OI and CI were equally effective in relaxing muscle. In addition, IJPs evoked by each of the inhibitors firing in high-frequency stimulus trains relaxed muscle to a comparable extent (Fig. 6B). Similar results were observed in two other preparations.

CI and OI are equally effective at inhibiting excitatory neurotransmission

In a series of classic experiments in the crayfish opener muscle, Dudel and Kuffler showed that stimulating the OI motoneuron within a few milliseconds of the excitor OE depressed the EJPs and that this phenomenon is due in part to presynaptic inhibition (Dudel and Kuffler, 1961). Repeating these experiments in the lobster demonstrates similar results: EJPs are reduced in size when OI is stimulated shortly before OE (Fig. 7). To test whether CI also inhibits excitatory neurotransmission, identical stimulation protocols were used to stimulate OE in sequence with either OI or CI, and the magnitude of the inhibition of the EJP was measured as a function of the interstimulus delay.

If stimulated within a short time before OE, both OI and CI inhibit synaptic transmission by the excitatory motoneuron OE by depressing the amplitude of the EJP (Fig. 8A). OI and CI are equally powerful in inhibiting excitatory neurotransmission, suppressing the size of the EJP by >60%, on average, at the shortest interstimulus intervals (Fig. 8B–D). Maximum inhibition by both inhibitory motoneurons occurs at interstimulus intervals of <60 ms, and recovery from inhibition is observed when the interstimulus intervals approach 300–400 ms. [Note that the duration of inhibition lasted much longer in these experiments at 2°C than the time course of 8–10 ms reported for the earlier crayfish experiments at 15°C (Dudel and Kuffler, 1961).] The prolonged nature of inhibition in the lobster experiments is probably the result of the long time course of synaptic responses at cold temperatures.

In some of the experiments summarized in Fig. 8, stimulation of OI prior to OE completely suppressed the excitatory synaptic response. Fig. 9 shows an example of a muscle fiber in which the synaptic response to OE was inhibited completely when OI was stimulated within 45 ms

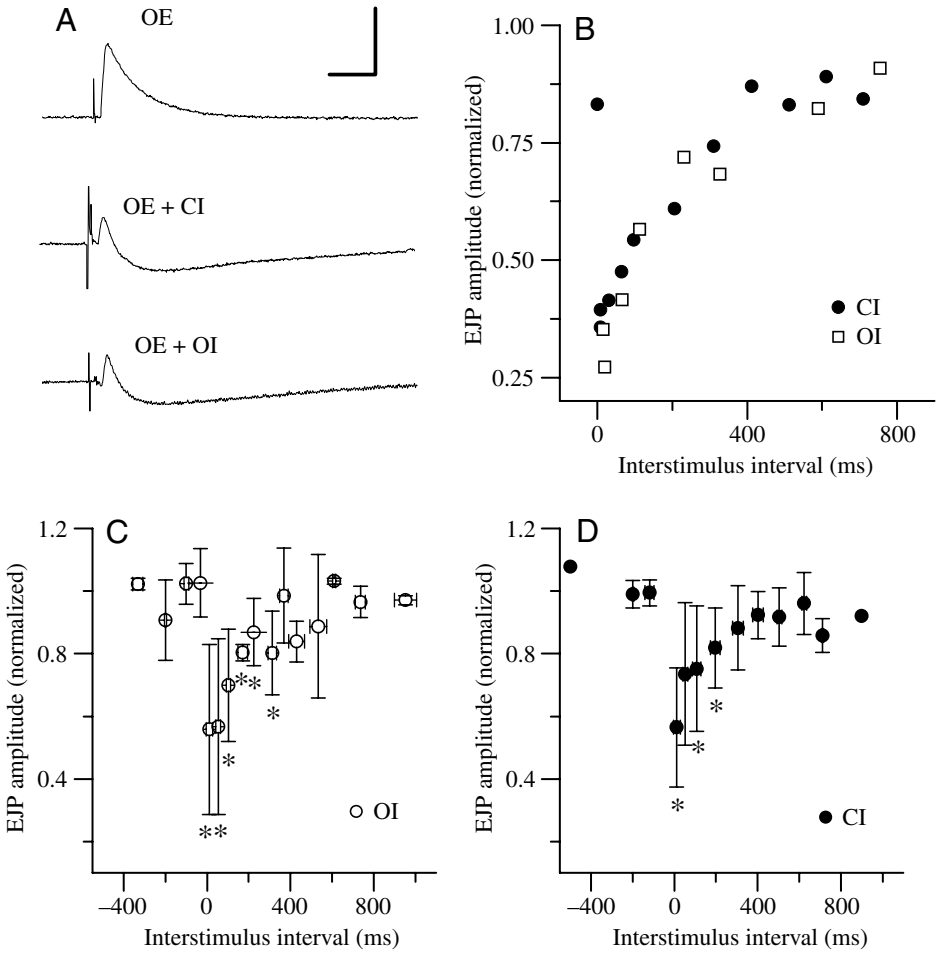

Fig. 8. Inhibition of excitatory transmission by the opener inhibitor (OI) and the common inhibitor (CI). (A) Examples of synaptic responses of a single muscle fiber to stimulation of the opener excitor (OE) alone, CI and OE (interstimulus delay 8.9 ms) and OI and OE (interstimulus delay 15.1 ms). Scale bar, 2 mV, 200 ms. (B) EJP amplitude plotted as a function of interstimulus interval between OE and each of the two inhibitors. All measurements are from a single muscle fiber; excitatory junction potential (EJP) size is normalized to its size in control trials where only OE was stimulated. (C,D) EJP amplitude, normalized to control, plotted as a function of the interstimulus delay between OE and OI (C, *N*=14 fibers) and OE and CI (D, *N*=8 fibers). Symbols represent the mean values, vertical error bars represent standard deviations of the means, and horizontal error bars indicate the range of interstimulus intervals in each bin. *Data are significantly different ($P<0.05$) from controls where the excitatory axon was stimulated prior to the inhibitory axon.

prior to OE stimulation. Such strong inhibition was rare; we observed complete inhibition of the EJP by OI in four out of 40 experiments at short (<50 ms) interstimulus intervals. By contrast, complete inhibition of the EJP by CI was not observed in 18 experiments at similarly short interstimulus intervals.

Whereas the preceding analysis of EJP amplitudes clearly demonstrates inhibition by both OI and CI, a more sensitive indicator of the degree of inhibition might be to analyze the change in shape of the EJP, rather than simply its amplitude. Fig. 10 shows examples of inhibition of the EJP by both OI and CI at 2°C. In both cases, analysis of the area under the curve of the EJP reveals a higher degree of inhibition than does the analysis of the amplitude of the EJP. To compare these two methods of analysis, we measured both the area under the EJP as well as EJP amplitude in multiple recordings where the delay between the EJP and the IJP was in the range 0–100 ms and compared these measurements to those for the respective control EJPs (Table 2). In 21 examples of presynaptic inhibition by OI, the inhibition measured as the change in area under the EJP was nearly twice that measured as inhibition of EJP amplitude (ratio inhibited/control=1.91). Similar results were obtained in 12 examples of recordings showing presynaptic inhibition by CI (ratio inhibited/control=2.01). Since depolarization directly gates calcium influx in crustacean muscle, changes in the overall shape of the EJP are likely to be critical for excitation–contraction coupling.

Table 2. *Inhibition of the excitatory junction potential (EJP) measured as a change in area and amplitude of the EJP*

Inhibitor	Muscle fibers (*N*)	Degree of inhibition (%)	
		Area (%)	Amplitude (%)
CI	12	51.3±5.7	28.1±6.0
OI	21	60.0±4.0	33.0±3.9

Values are means ± s.d. The degree of inhibition of the EJP was determined by measuring the area or amplitude of EJPs in intracellular recordings in which an inhibitor (CI or OI) was stimulated 0–110 ms prior to stimulation of the excitor (OE). These values were then compared with those recorded from the same fiber when only OE was stimulated.

OI and CI are equally effective in inhibiting contractions by OE

To compare the effectiveness of both inhibitors in reducing muscle contractions triggered by OE, short stimulus trains were delivered to the axon of OE in the presence and absence of identical trains simultaneously delivered to one of the

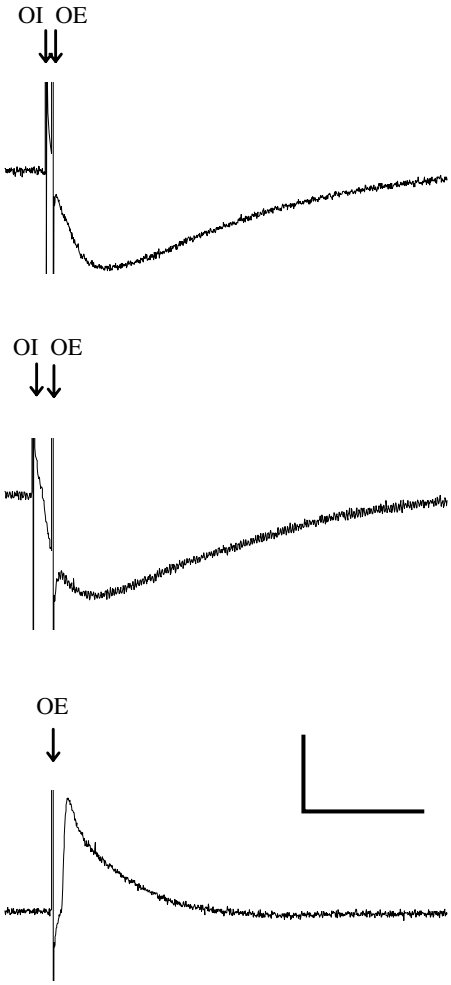

Fig. 9. The opener inhibitor (OI) can completely suppress the synaptic response to the opener excitor (OE). Three recordings are shown from the same muscle fiber; the top two show the result of stimulating OI 18.4 ms and 46.8 ms, respectively, before stimulation of OE. The bottom trace shows the control response to OE stimulation. Scale bar, 1 mV, 200 ms.

inhibitors. Fig. 11 is representative of three experiments in which we observed that contractions evoked by OE can be completely abolished by simultaneous firing of either OI or CI. Although the intracellular record reflects physiological activity in a single muscle fiber while the contraction is measured from many fibers, two aspects of these recordings are notable. Inhibition of muscle contraction is profound, being 100% effective for relatively few inhibitory stimuli in either inhibitor (provided they are optimally timed with respect to the excitatory stimuli). In addition, the contractions failed to develop even though neither inhibitor completely abolished the EJPs. Assuming the intracellular record from this single muscle fiber is representative of the majority of muscle fibers, the records suggest that the inhibited EJPs are not large enough or do not facilitate sufficiently to generate the contraction.

Discussion

In this report, we show electrophysiological evidence that the common inhibitor CI innervates fibers along the entire length of the lobster dactyl opener muscle and therefore shares the postsynaptic targets of the two other innervating neurons, the specific excitor OE and the specific inhibitor OI. Further, the physiological effects of CI in this neuromuscular system mimic those of OI: both inhibitors relax muscle, both depress the EJPs triggered by the excitatory motoneuron OE, and both depress OE-stimulated muscle contractions. At the low stimulus frequencies we examined, CI and OI are approximately equally powerful.

These results are surprising, given that previous work in the crayfish dactyl opener failed to find synaptic responses to CI in any but the most medial of the longitudinally oriented fibers of the most proximal end of the muscle (Wiens, 1985). In addition, in the crayfish, the physiological consequences of CI stimulation are weak compared with those of OI: CI innervates fewer fibers, the IJPs elicited by CI are smaller, and CI is considerably less effective at abolishing contractions elicited by the excitor OE (Wiens, 1985; Wiens, 1993). A previous study in the lobster reported similar results; IJPs resulting from OI stimulation were stronger initially and maintained or increased their effectiveness more effectively than those evoked by CI (Wiens, 1990). However, only muscle fibers

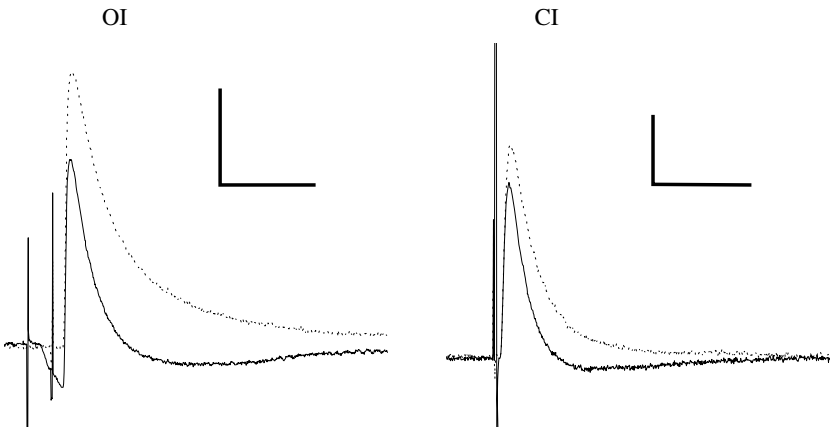

Fig. 10. Excitatory junction potentials (EJPs) inhibited by the opener inhibitor (OI) (left: interstimulus interval 52 ms, OI precedes OE) and by the common inhibitor (CI) (right: CI and OE stimulated simultaneously) superimposed on the EJPs recorded in the absence of inhibitory stimuli (broken lines). In these examples, OI inhibited the area under the EJP by 75.9% and the amplitude of the EJP by 29.7%. CI inhibited the area under the EJP by 49.8% and the amplitude of the EJP by 16%. Scale bar on left, 2 mV, 200 ms; scale bar on right, 1 mV, 200 ms.

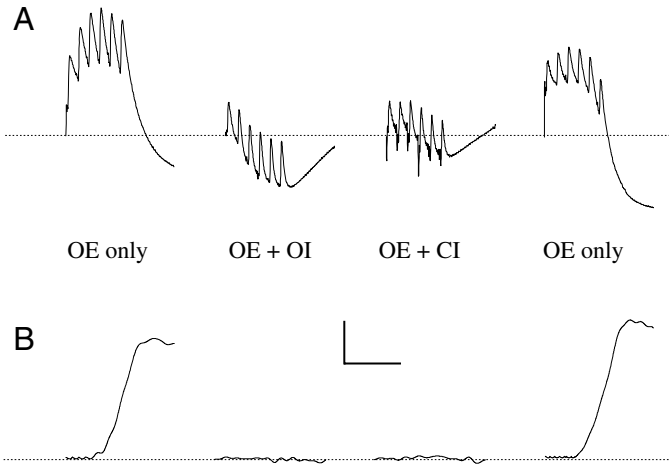

Fig. 11. The opener inhibitor (OI) and the common inhibitor (CI) are similarly effective in inhibiting synaptic and contractile responses to stimulation of the excitor OE. (A) Intracellular recordings of synaptic responses to identical stimulus trains (10 Hz, 600 ms) delivered to OE alone, OI and OE (stimulated simultaneously), CI and OE (stimulated simultaneously) and OE alone. Both inhibitors inhibit excitatory junction potential (EJP) amplitude and hyperpolarize muscle slightly. The broken line illustrates resting membrane potential. (B) Tension recordings corresponding to stimulus trials above. Note that the muscle contracts in response to stimulation of OE alone but fails to contract if either inhibitor is stimulated. The broken line indicates resting level of muscle tension in the absence of stimulation. Scale bar, 2.5 mg, 100 mg, 500 ms.

located in the proximal third of the lobster opener were sampled for CI innervation, and synaptic responses to stimulus trains were reported for only two muscle fibers.

Our results demonstrate that in the lobster dactyl opener, the generality that OI is a stronger inhibitor than CI does not hold. While overall the IJPs triggered by CI are smaller, in one third of fibers (11 out of 34) CI's IJPs were in fact larger than those of OI. Further, although we did not systematically examine the facilitation properties of the two inhibitors, it is clear that IJPs elicited by CI can show more facilitation than those elicited by OI (for example, see Fig. 6B). The two inhibitors are similarly powerful in inhibiting the EJPs stimulated by OE (Fig. 8), and both inhibitors, firing at relatively low frequency, can abolish muscle contractions stimulated by OE (Fig. 11). Finally, both OI and CI, firing either once or in trains, can relax the dactyl opener muscle to approximately the same extent (Fig. 6).

The reasons for the discrepancies between our findings and those of others are unclear, however we note that the low temperature used in our experiments favors large IJPs because the resting potentials of the muscle fibers at 2°C are depolarized relative to their values at 10–11°C, where the IJPs reverse polarity (see Table 1). At 2°C, our ability to detect hyperpolarizing synaptic responses is relatively enhanced because the driving force for the IJPs is relatively large at cold temperatures. Previous crayfish studies have been done at room temperature, where the resting membrane potential is close to the equilibrium potential for the chloride ions and inhibitory synaptic signals can be relatively small or at their reversal potential. Under these conditions, it is possible that inhibitory synaptic responses may not be detectable in the background noise of the recording.

It is important to note that temperatures as cold as 2°C are in fact physiologically normal for lobsters living in the wild. By affixing temperature loggers to *Homarus* living in the Gulf of Maine, Cowan and co-workers demonstrated that this species lives in extremely cold (0–6°C) water temperatures throughout the six months of winter and early spring (Cowan et al., in press).

Because we performed our experiments in the cold (2°C), two particular physiological issues deserve mention. First, the resting muscle tension relieved by CI and OI (see Fig. 6) appears to be relatively strong in the cold (M.K.W., unpublished observations), possibly because the muscle fibers are sufficiently depolarized to mediate a resting calcium influx. However, little is known about the generation of resting muscle tension in this or any other crustacean muscle, and the mechanisms by which inhibitors relax muscle tension remain to be elucidated. Second, it is possible that cold temperatures compromise the safety factor for action potential conduction by slowing the kinetics of ion channel activity in axonal membranes. At 2°C, we observe that the effectiveness of presynaptic inhibition is >60% for each of the inhibitors (Fig. 8), however presynaptic inhibition might be even stronger at warmer temperatures. Future experiments will address whether the strength of presynaptic inhibition in the lobster might be temperature dependent.

Finally, our observation that one of the three axons innervating the dactyl opener muscle is of much smaller diameter than the others (Fig. 5) may explain why the triple innervation of this muscle in lobster has been largely overlooked. Previous authors described two major axons in cross-sections of fixed dactyl opener nerve and assumed that the smaller axon profiles were branches of the two main axons OI and OE (Kravitz et al., 1963). In our hands, most methylene blue preparations of dactyl opener muscle show two large axons over most of the extent of the muscle, however evidence for three can be observed with close examination (see Fig. 5). The technical problem of underestimating the number of methylene blue-stained axons if two axons run in close proximity or one lies over another has been noted previously in a discussion of the triple innervation of the crayfish extensor muscle (Van Harreveld and Wiersma, 1937).

What distinctive physiological roles are served by each of the inhibitors?

Our finding that CI can be as powerful as OI in several physiological assays at a physiologically relevant temperature is important in understanding the functional roles of these two inhibitors. Previously, it was thought that the physiological roles of the common and specific inhibitors may be distinct, in that the inhibition by CI is more general in scope (reaching multiple muscles) and weaker in nature. By contrast, the physiological roles of specific inhibitors (such as OI) have

been strongly associated with inhibiting the output of the specific excitors, which they powerfully inhibit and with which they share all innervation targets. In a review, Wiens summarized the distinction between the specific and common inhibitors in decapod crustaceans by noting that, in contrast to the common inhibitor, specific inhibitors '*innervate all fibers in their target muscles powerfully and can totally eliminate any contraction in these muscles*' (Wiens, 1989). However, our data would suggest that such a view requires revision, as the common inhibitor CI fits this description as well as the specific inhibitor OI in the lobster dactyl opener neuromuscular system.

Other neuromuscular systems where the physiological ramifications of dual inhibitory innervation have been extensively studied include those of the brachyuran crab *Eriphia*. In the crab, where the dactyl opener is composed of both tonic and phasic fibers, CI inhibits the tonic fibers strongly and the phasic fibers weakly, and this pattern is common to each of the seven limb muscles in the crab (Wiens et al., 1988). By comparison with the specific inhibitors to the crab muscles, CI is usually reported to have weaker physiological effects. For example, in the crab, CI exerts little presynaptic inhibition on the excitor innervating the stretcher muscle whereas the specific inhibitor to the stretcher exhibits both presynaptic and postsynaptic inhibition (Atwood and Bittner, 1971); in this system at firing frequencies below 70 Hz CI is considerably less effective than the specific inhibitor at reducing EJP size. In addition, in electromyograms of the crab, OI nearly completely suppressed the electrical response of the muscle to OE, but CI rarely did so (Ballantyne and Rathmayer, 1981). Interestingly, in the crab flexor, as in the lobster opener (see Fig. 6), short bursts of firing activity in CI in isolation can effectively relax muscle tension (see fig. 4 in Wiens and Rathmayer, 1985).

Given that the peripheral effects of OI and CI are very similar in the lobster dactyl opener, one speculation is that any distinctions between their physiological functions *in vivo* must be due to differential firing activity at the level of the CNS. A previous examination of central control of dactyl movement evaluated efferent neural activity to both the dactyl opener and the closer in the crayfish claw during reflex activity. The findings suggested that OI and CI may have mutually inhibitory connections, ensuring they will not fire simultaneously (Wiens and Gerein, 1975). Interestingly, the data also indicated that CI might inhibit OE centrally, whereas we have demonstrated that CI powerfully inhibits OE peripherally. However, it was not clear from the earlier study under what conditions firing of one inhibitor might be favored over that of the other, nor was it known that CI, in fact, makes synapses on the opener muscle as well as the closer. Thus, a definitive comparison between the roles of CI and OI in moving the dactyl is lacking. The most comprehensive examination of the central activity of CI has been in a chronic recording of CI efferent activity during locomotion in the crab (Ballantyne and Rathmayer, 1981). Firing tonically at low frequencies, CI shaped muscle contractions stimulated by bursts of activity in OE such that contractions became more phasic in nature, relaxing more quickly.

Under the stimulus conditions we tested (using single inhibitory stimuli and short stimulus trains) we did not observe differences in the physiological impact of OI as compared with CI in the lobster dactyl opener neuromuscular system. However, we cannot exclude the possibility that differences exist under other stimulus paradigms, such as when the inhibitors fire in prolonged or high-frequency stimulus trains. In addition, there may be differences in the membrane composition of the presynaptic terminals of each of the inhibitors that could be important physiologically. For example, in the crayfish dactyl opener it has been shown that different classes of GABA receptors mediate presynaptic inhibition by OI depending on the frequency of firing (Fischer and Parnas, 1996). It is unknown whether the terminals of CI operate similarly. Inhibitory terminals on crayfish dactyl opener stain with antibodies to GABA (Msghina and Atwood, 1997) as well as antibodies to mammalian $GABA_A$ receptors (Feinstein et al., 2003), however the authors assumed that staining corresponded exclusively to terminals of OI rather than CI. We would predict that terminals from both inhibitors would stain for GABA and GABA receptors in the lobster opener, consistent with our observation that synaptic responses to both inhibitors reverse at the same membrane potential.

In summary, CI and OI share innervation targets in the lobster dactyl opener and are comparable in terms of their physiological effects in relaxing muscle, inhibiting excitatory synaptic responses, and reducing neurally evoked muscle contractions. Future studies should elucidate whether and how these two inhibitors are differentially activated by the CNS. As CI also provides the only source of inhibition to the opposing dactyl closer muscle, understanding the relative roles of OI and CI motoneurons will be important for understanding the neural control of both of the muscles that move the dactyl.

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References

Atwood, H. L. (1972). *Crustacean Muscle.* New York: Academic Press.

Atwood, H. L. and Bittner, G. D. (1971). Matching of excitatory and inhibitory inputs to crustacean muscle fibers. *J. Neurophysiol.* **34**, 157-170.

Ballantyne, D. and Rathmayer, W. (1981). On the function of the common inhibitory neurone in the walking legs of the crab, *Eriphia spinifrons*. *J. Comp. Physiol.* **143**, 111-122.

Bykhovskaia, M., Hackett, J. T. and Worden, M. K. (1999). Asynchrony of quantal events in evoked multiquantal responses indicates presynaptic quantal interaction. *J. Neurophysiol.* **81**, 2234-2242.

Bykhovskaia, M., Polagaeva, E. and Hackett, J. T. (2004). Mechanisms underlying different facilitation forms at the lobster neuromuscular synapse. *Brain Res.* **1019**, 10-21.

Cowan, D. F., Watson, W. H., Solow, A. and Mountcastle, A. (in press). Thermal histories of brooding lobsters, *Homarus americanus*, in the Gulf of Maine. *Mar. Biol*.

Dudel, J. and Kuffler, S. W. (1961). Presynaptic inhibition at the crayfish neuromuscular junction. *J. Physiol.* **155**, 543-562.

Feinstein, N., Fritschy, J. M. and Parnas, I. (2003). Presynaptic membrane of inhibitory crayfish axon terminals is stained by antibodies raised against mammalian GABA(A) receptor subunits alpha3 and beta(2/3). *J. Comp. Neurol.* **465**, 250-262.

Fischer, Y. and Parnas, I. (1996). Differential activation of two distinct mechanisms for presynaptic inhibition by a single inhibitory axon. *J. Neurophysiol.* **76**, 3807-3816.

Govind, C. K. (1995). *Muscles and their Innervation.* San Diego: Academic Press.

Kravitz, E. A., Kuffler, S. W., Potter, D. and van Gelder, N. M. (1963). Gamma-aminobutyric acid and other blocking compounds in crustacea. II. Peripheral nervous system. *J. Neurophysiol.* **26**, 729-738.

MacDermott, A. B., Role, L. W. and Siegelbaum, S. A. (1999). Presynaptic ionotropic receptors and the control of transmitter release. *Annu. Rev. Neurosci.* **22**, 443-485.

Marmont, G. and Wiersma, C. (1938). On the mechanism of inhibition and excitation of crayfish muscle. *J. Physiol.* **93**, 173-193.

Mearow, K. and Govind, C. K. (1986). Neuromuscular properties in the serially homologous lobster limbs. *J. Exp. Zool.* **239**, 197-204.

Msghina, M. and Atwood, H. L. (1997). Distribution and morphology of inhibitory innervation in crayfish (*Procambarus clarkii*) limb and abdominal muscles. *Cell Tissue Res.* **290**, 111-118.

Parnas, I., Rashkovan, G., Ravin, R. and Fischer, Y. (2000). Novel mechanism for presynaptic inhibition: GABA(A) receptors affect the release machinery. *J. Neurophysiol.* **84**, 1240-1246.

Van Harreveld, A. (1939). The nerve supply of doubly and triply innervated crayfish muscles related to their function. *J. Comp. Neurol.* **70**, 267-284.

Van Harreveld, A. and Wiersma, C. (1937). The triple innervation of crayfish muscle and its function in contraction and inhibition. *J. Exp. Biol.* **14**, 448-461.

Vyshedskiy, A. and Lin, J. W. (2000). Presynaptic Ca^{2+} influx at the inhibitor of the crayfish neuromuscular junction: a photometric study at a high time resolution. *J. Neurophysiol.* **83**, 552-562.

Vyshedskiy, A., Delaney, K. R. and Lin, J. W. (1998). Neuromodulators enhance transmitter release by two separate mechanisms at the inhibitor of crayfish opener muscle. *J. Neurosci.* **18**, 5160-5169.

Wiens, T. J. (1985). Triple innervation of the crayfish opener muscle: the astacuran common inhibitor. *J. Neurobiol.* **16**, 183-191.

Wiens, T. J. (1989). Common and specific inhibition in leg muscles of decapods: sharpened distinctions. *J. Neurobiol.* **20**, 458-469.

Wiens, T. J. (1990). The inhibitory innervation of the walking leg of the lobster, *Homarus americanus*. *J. Comp. Physiol. A* **167**, 43-50.

Wiens, T. J. (1993). The closer muscle is a second target for the stretcher inhibitor motoneuron of the crayfish's thoracic limbs. *J. Comp. Physiol. A* **173**, 435-444.

Wiens, T. J. and Gerein, G. L. (1975). Cross connections among crayfish claw efferents. *J. Neurophysiol.* **38**, 909-921.

Wiens, T. J. and Rathmayer, W. (1985). The distribution of the common inhibitory neuron in brachyuran limb musculature. I. Target muscles. *J. Comp. Physiol. A* **156**, 305-313.

Wiens, T. J., Maier, L. R. and Rathmayer, W. (1988). The distribution of the common inhibitory neuron in brachyuran limb musculature. II. Target fibers. *J. Comp. Physiol. A* **163**, 651-664.

Worden, M. K., Kravitz, E. A. and Goy, M. F. (1995). Peptide F1, an N-terminally extended analog of FMRFamide, enhances contractile activity in multiple target tissues in lobster. *J. Exp. Biol.* **198**, 97-108.

Worden, M. K., Bykhovskaia, M. and Hackett, J. T. (1997). Facilitation at the lobster neuromuscular junction: a stimulus-dependent mobilization model. *J. Neurophysiol.* **78**, 417-428.

Zucker, R. S. (1973). Changes in the statistics of transmitter release during facilitation. *J. Physiol.* **229**, 787-810.